Possible odour-mediated attraction of flies to *Bacillus subtilis* NRS-762 stationary phase culture

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Conflicts of Interest
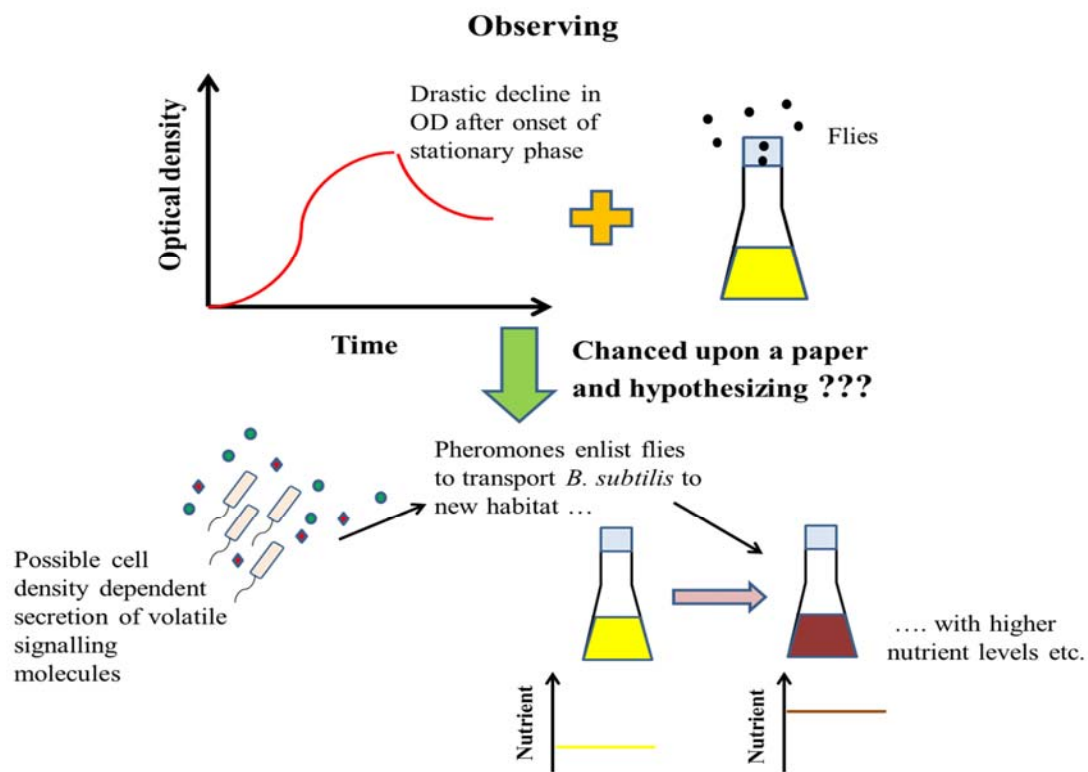
The author declares no conflicts of interest.

Author's contributions

The paper by Wood and coworkers helped the author connect the dots and formulate a hypothesis for explaining an observation (flies on the cotton plugs of *Bacillus subtilis* culture) thought to be unimportant or mundane. No direct molecular data supports the postulation presented. The hypothesis (interpretation) was written up for other researchers to take the work further.

Supporting information

A supplementary file containing an outline of possible future work addressing questions that emanate from the reported preliminary observations is available.

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Graphical abstract

Observing

Drastic decline in OD after onset of stationary phase

Optical density

Time

Flies

Chanced upon a paper and hypothesizing ???

Possible cell density dependent secretion of volatile signalling molecules

Pheromones enlist flies to transport *B. subtilis* to new habitat …

…. with higher nutrient levels etc.

Nutrient

Nutrient

Short description

Chance encounter with a journal article helped put into perspective serendipitous observations otherwise thought to be mundane. Specifically, drastic decline in optical density of odourous stationary phase *Bacillus subtilis* NRS-762 culture coincident with flies moving on or flying around the cotton plugs of shake-flasks, suggested possible interkingdom signalling mediated by volatile compound(s) secreted by the bacterium. Possible ecological significance of such signalling include the enlistment of flies as transport vectors for dispersing the bacterium to more favourable ecological niches, while the bacteria protect the host from colonization by invasive species and provide it with metabolic benefits. More important, such vector-mediated long-range dispersal is ideally-suited for preserving the clonal population in the event of irreversible decline in habitability at the original site, and may exist in parallel to other strategies such as induction of dormancy or spore formation for *in situ* response to short and long-term (but reversible) environmental fluctuations. Such a comprehensive repertoire of signalling and metabolic remodelling mechanisms, each targeted towards differing severity of environmental fluctuations on different timescales, may underpin and explain the evolutionary resilience of members of the Bacteria domain over geological eons.

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Highlights

1) Drastic decline in optical density of *Bacillus subtilis* stationary phase culture co-occurred with presence of flies and emanation of odour from culture.

2) Increase in odour pungency negatively correlated with cell density; suggesting the production of volatile signalling molecules by remaining cells in a "SOS" response mediated by a positive feedback loop.

3) Lack of cellular debris after autoclave decontamination suggested non-completion of sporulation pathway in *B. subtilis*.

4) Proposed conceptual model posits the secretion of volatile interkingdom signalling molecules by *B. subtilis* that enlist flies for long-range dispersal to habitats with more favourable conditions.

5) Corollary from the model suggested the existence of a two-pronged stress response mechanism, where sporulation and dormancy pathways cater to reversible changes in environmental conditions, while long-range dispersal aids in clonal population survival in the event of irreversible decline in habitability.

Abstract

Signalling helps connects different organisms in the biosphere. This research note describes the possible concentration-dependent odour mediated attraction of flies to stationary phase aerobic liquid cultures of *Bacillus subtilis* NRS-762 maintained on an open orbital shaker at 25 °C. Greater odour pungency correlated with more intense "foraging" attempts, which suggested the compounds' possible behaviour modifying effects. Additionally, co-occurrence of optical density decline and increase in odour pungency suggested volatile compound(s) secretion might be a cell survival response mediated by a cell density-based signalling mechanism. Flies were not attracted to odourous stationary phase cultures of other common bacteria (*Escherichia coli* DH5α, *Pseudomonas aeruginosa* PRD-10, and *Pseudomonas protegens* Pf-5, which suggested species-specificity of the volatile compound(s). Altogether, volatile compound(s) might serve as interkingdom messengers to enlist flies for dispersing *B. subtilis* to habitats with more favourable conditions in coping with possible irreversible decline in habitability, and operate in parallel with other *in situ* mechanisms such as cannibalism and sporulation that help *B. subtilis* ride out short- and long-term environmental fluctuations, respectively. Interested researchers are invited to build upon the preliminary findings.

Keywords: cell signalling; species dispersal; transport vector; behavior modification; stationary phase; volatile compound; microbial ecology; colonization; microbiome;

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1. Introduction

Individual organisms in the biosphere are part of a broader community and interact with each other – both within and across species – through a variety of chemical and physical cues. The web of interactions promote community resilience and help deliver important ecosystem services [1]. Interactions between organisms can take many forms and fall along the continuum from antagonistic to synergistic; for example, commensalism, competition, predation, no interaction, mutualism, and amensalism [2,3]. Additionally, possibilities exist for multiple interactions to simultaneously occur between a species pair. For example, two species may compete for a particular substrate (i.e., competition), but one organism may generate a useful metabolite that the other is unable to produce.

Bacteria secrete a variety of biomolecules that mediates processes and coordinate behavior at the cellular and population levels. Besides the diffusible molecules that partake in signaling processes in liquid and solid medium culture, bacteria also release a variety of volatile compounds [4], where antimicrobial activity is the most direct observable effect. For example, biogenic gaseous ammonia modulates antibiotic resistance in a range of Gram-negative and Gram-positive bacteria [5,6]. Additionally, volatile compounds have also been shown to exert antimicrobial activity towards a broad range of fungi [7-9]. Another study profiles the volatile organic compounds secreted by 200 bacterial isolates and reveals that many of the volatiles exert nematicidal activity [10]. Thus, accumulated knowledge suggests that volatile organic compounds secretion may have been shaped by evolutionary forces as a form of protection for bacteria against worms and fungus etc.

Besides antimicrobial activity, volatile compounds also mediate intercellular communication between bacteria within and across species, although much remains to be known concerning the volatile compounds' roles in mediating bacteria cell-cell communication [6]. Specifically, the compounds potentiate a variety of physiological and metabolic processes in individual cells that, in aggregate, translate into population level behavior [11]. For instance, diffusible small molecules serve as communication agents for coordinating diverse activities between various bacteria in the root rhizosphere. Characterization of the long-range effects of 14 gaseous volatile compounds secreted by *Escherichia coli* also reveal their myriad effects on biofilm formation, motility pattern and antibiotic resistance in *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* [5].

In order for signaling to elicit the desired phenotype, information must be transmitted via a cascade of "carrier" molecules and molecular processes – from the signalling molecule, to

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conformational changes of receptors upon ligand binding at the cell membrane, to intracellular secondary messengers, which finally, either alter protein production/degradation post-transcriptionally, or modulate gene expression directly. In the latter case, promoters in *B. subtilis* are known to be activated by specific volatile compounds in spoiled meat [12]. From a genetic and evolutionary perspective, volatile compound dependent modulation of gene expression holds important implications in understanding how odours serve as a selective force in shaping the evolution of gene regulatory networks in bacteria and, more important, the ecological context of such a chemical compound-gene transcription coupling. Taken together, the overarching theme running through microbial intercellular communication is the use of small diffusible chemical compounds as signalling molecules that induce a specific response through activating targeted genes or pathways.

Though microbe-microbe communication have received intense research interest, and have yielded numerous interesting discoveries, do microbes communicate with "higher" organisms? Answer to the question depends on the nature and purpose of interkingdom signaling; for example, exchanges of metabolites or bacterial secretion of molecules that exert a physiological effect on target organisms. Recent research reveals that various microorganisms (e.g., fungus, bacteria, virus and archaea) are resident partners – both on and within - many higher organisms and, more important, modulate host's gene expression and metabolism [13-15]. In particular, high degree of concordance in microbiota diversity and community structure of different fungus-cultivating insects ranging from ants to beetles has been observed; thereby, suggesting that specific consortia of microbes is necessary for conferring particular metabolic capabilities, in this case, either facilitating fungus cultivation or catabolizing the nutrients and metabolites generated [15]. Thus, lines of communication, both physical (cell-cell contact) and chemical (diffusible molecules), should exist between microorganisms and host cells. Indeed, studies have illuminated that complex communication networks exist between host cells and resident microbes – and such exchange of signalling and metabolic factors play important roles in modulating host's physiology, immune functions, metabolism, and even cognitive processes [16,17]. Other studies have delineated physiological effects of bacteria secreted volatile compounds such as plant growth and defense, and colonization of nematode hosts [6,18-20]. Perhaps, more intriguingly, RNAi mediated production of a neuropeptide allows recombinant *E. coli* – bound to *Caenorhabditis elegans* via an adhesin – to modulate the social feeding behavior of the worm; specifically, inducing the formation of clumps comprising dozens to hundreds of individual cells [17]. Thus, beyond a pure communication function, interkingdom messaging molecules may exert behavior modification activity in the host at the organismal level through hitherto unknown mechanisms.

While diffusible molecules facilitates communication between bacteria and host cells, what about cases where the communication needs of the bacterium or microbe extend beyond the

immediate surroundings of the colony or the host-microbe interface? Such a mode of communication could find use, for example, in enabling bacteria to signal for help during times of nutritional stress. Specifically, if the nutritional or environmental conditions at the original niche becomes unfavourable, are there means by which bacteria are able to signal for help - for example, secreting volatile interkingdom signalling molecules – to attract other species such as insects for facilitating their transport to alternative habitats with more favourable conditions for growth and colonization?

Various insects serve as vectors for microbes and parasites important in human diseases such as malaria, dengue fever, and yellow fever etc. Quorum-sensing is known to mediate communications between bacteria and higher organisms such as insects [21]. Extensive research over decades have characterized the directionality, mechanistic underpinnings, and molecular effectors of such parasitic and mutualistic relationships [22], where an insect either serve as a passive transporter of the microbe or, plays a more active role in helping complete part of the parasites' life cycle (e.g., malaria). In fact, many insects rely on symbiotic microbes for growth, survival and reproduction. Beeoove wasps, for example, cultivate antibiotic-producing *Streptomyces* for protecting their larvae against microbial pathogens [22]. On the other hand, bacteria are reported to affect various stages of host development [23]. Similar to humans and other mammals, insects' gastrointestinal (GI) tracts are also colonized by a community of diverse microbes, many of which are commensals and non-pathogenic [22,24,25]. Looking beyond the well-understood roles of insects as disease vectors, less focus is placed on understanding alternate mechanisms underpinning the colonization of the insects' gastrointestinal (GI) tracts by specific bacteria, besides the oft-noted roles of microbes facilitating host digestion of recalcitrant nutrients, or providing the host with essential vitamins.

In addition to providing beneficial nutrients and exchanging metabolites, microbes (in particular, bacteria) resident in insects' GI tract may also be "hitchhikers" on a ride between ecological niches. Particularly, given the significantly longer forage range of insects (particularly those air-borne) relative to that of most microorganisms, establishment of a commensal or neutral relationship between microbe and insect may help in dispersing the microbes to a wider variety of habitats; thereby, serving as a hedge for species or clonal population survival during severe nutritional stress, or expanding the colonization range of an invasive species. Dispersal for colony survival is particularly important given the non-homogenous distribution of nutrients, temperature and water across physical space. Hence, with the greater dispersal range of insect vectors and their acute sense of smell (useful for spotting nutritionally favourable locales), the chances that the bacterium would be dispersed to niches with good nutritional and environmental conditions are much better relative to prospects of waiting for favourable conditions to return at the original niche.

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Extending the above analysis, research has confirmed the secretion of volatile compounds by bacteria for attracting insects [26]. For example, headspace sampling (e.g., purge and trap) coupled with gas chromatography mass spectrometry (GC-MS) has identified a variety of natural products – including insect pheromones - in the headspace above a bacterial culture [27]. This indicates that volatile compounds secretion may be a mode of signaling and communication between different bacterial species, and between bacteria and their symbiotic insect host. Interestingly, the housekeeping protein, heat shock protein (GroEL) also mediates both mutualistic and pathogenic microbe-insect interactions [28]. Given that GroEL is highly conserved across species and mediates *in vivo* protein folding during heat stress, the finding suggests that signaling between organisms has a long evolutionary history. Another study reports the secretion of odourous compounds by *Proteus mirabilis* for attracting blow flies [26], which suggests that interkingdom signalling may be involved in dispersing bacterial species from unfavourable habitats to more favourable microcosms with greater nutrient abundance. More intriguingly, six fly attractants – phenol, NaOH, KOH, lactic acid, ammonia and putrescine – are shown to induce swarming motility in *P. mirabilis* mutants deficient in the motility mode via activating hitherto unknown pathways. Transposon mutagenesis further reveals six novel genes in *P. mirabilis* (*ureR*, *fis*, *hybG*, *zapB*, *fadE*, and *PROSTU_03490*) activated by the above fly attractants [26]. Since the odour compounds are secreted during stationary phase and activate swarming motility in *P. mirabilis*, possibility exists that the odour compounds are physiological signals activating cellular motility for searching new food sources in times of nutritional stress. Thus, the described research suggests a link between swarming motility in *P. mirabilis,* and interkingdom signaling between blow flies and the bacterium. The latter may be an intermediary step in the colonization of new ecological niches for expanding access to nutrients - and thus, greater survivability of the clonal population, particularly, in the event of irreversible deterioration in a microcosm's habitability.

Herein, this research note describes a serendipitous observation of flies moving on or flying around the cotton plugs of aerobic stationary phase cultures of *B. subtilis* NRS-762 (ATCC 8473) cultivated at 25 °C on an open orbital shaker. A strong pungent odour - reminiscent of that from rotten food – emanated from the flasks and was coincident with flies presence. Similar pungent odours were also detected in late stationary phase cultures of *B. subtilis* NRS-762 maintained at 30 and 37 °C in closed incubator orbital shakers – but the incubator doors probably prevented flies entry. Although stationary phase cultures of *E. coli* DH5α, *Pseudomonas protegens* Pf-5, and *P. aeruginosa* PRD-10, cultivated under identical conditions, also emanated odours (distinct from *B. subtilis*), no flies were observed around the cultures; thus, suggesting that *B. subtilis* secreted species-specific signaling molecules. Odour pungency also positively correlated with number of flies present on or around the cotton plugs, and more intense attempts at gaining "entrance" into the cotton matrix, the latter a possible sign of behavior modification induced by the volatile compounds. Finally, increased odour pungency was coincident with precipitous decline in OD_{600} during late stationary and death phases, which suggested a positive feedback loop linking cell

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density and odour compound biosynthesis that drove the production of more volatile compounds by remaining cells of a rapidly declining population.

Possible reasons underlying the observed phenomenon would be discussed via a conceptual model. Specifically, upon entering stationary phase and encountering nutritional stress, *B. subtilis* synthesized volatile compound(s) for signalling long-range dispersal to new ecological niches, with flies as a possible transport vector. Biosynthesis of the volatile compound(s) was hypothesized to be mediated by a cell density-based signal transduction system where declining population size induced greater secretion of volatiles in a positive feedback loop. Activation of the long-range dispersal mechanism likely occurred in parallel with other more well-known nutritional stress responses in *B. subtilis* (such as cannibalism and sporulation) evolutionarily designed for coping with short to long-term fluctuations in environmental conditions. The above long-range dispersal of *B. subtilis* to new microcosms likely constituted a hedge against irreversible decline of the original niche's habitability. With different cellular survival mechanisms capable of coping with environmental changes occurring on a continuum of timescales, a comprehensive sensing, signalling and response repertoire might have evolved in *B. subtilis* and, by extension, across other branches of the Bacteria domain. Interested researchers may test the inter-related hypotheses presented in the model, and build upon the preliminary observations.

2.   Materials and Methods

2.1   *Bacterial strain*

B. subtilis NRS-762 was purchased from Agricultural Research Service (ARS) culture collection – and maintained in the lab as a glycerol stock culture without genetic modifications or, underwent selection processes for obtaining target adaptations. Nevertheless, given that the laboratory strain had been sub-cultured on numerous occasions, possibilities exist that mutations (some of which introducing phenotypic or metabolic changes) could have occurred, but remained unnoticed in the absence of genome sequencing. According to ATCC and ARS, *B. subtilis* NRS-762 was isolated from ropy bread, but further details on the place of origin of the strain, or whether it was domesticated are not available. There is no sequenced genome for this *B. subtilis* strain.

2.2.   *Cell cultivation*

Glycerol stock cultures of *B. subtilis*, maintained at -70 ℃ prior to inoculation, were used in growing a seed culture in 100 mL Difco's LB Lennox medium (composition in g/L: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0), in 250 mL borosilicate glass shake-flasks under aerobic

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conditions at ~25 °C and 250 rpm on an open orbital shaker (IKA KS-260 Basic, Germany). After 8 hours of cultivation, 1 mL of seed culture served as inoculum for 100 mL of fresh LB medium in experiment cultures conducted under identical conditions. Cultivations were in batch mode with no nutrient supplementation. Fresh cotton plugs were used for all cultures and fit tests were conducted to ensure proper sealing of each flask of medium. Three biological replicates were used in each experiment. The orbital shaker was positioned on a lab bench away from heat sources, volatile chemicals, or incubator shakers housing other bacterial cultures, to reduce the possibility that volatile compounds emanating from other cultures or sources might affect the observations. At specific time-points, aliquots (5 mL) of cultures were withdrawn for measuring optical density at 600 nm (OD_{600}) via a Shimadzu Biospec-Mini UV-Visible spectrophotometer. Aliquots were diluted with deionized water if the absorbance exceeded 1. pH of the aliquots were measured using a Mettler Toledo AE-100 pH meter outfitted with an Orion pH electrode.

3. Results and discussion

3.1 Decline in optical density and presence of flies on cotton plugs

After a short lag phase of 2.5 hours, *B. subtilis* exhibited good growth; achieving an optical density (OD_{600}) of 5.4 after 22.5 hours of cultivation (Fig. 1). Nevertheless, there was a precipitous drop in OD_{600} thereafter; decreasing to 3.8 and 2.5 at 26 and 38 hours post-inoculation. The exact reason(s) for the drastic decrease in OD_{600} was not known; however, possible causes might include induction of lytic cycle by prophages or activation of autolysin upon exposure to nutritional stress during late stationary phase. Additionally, no visible cell debris or fragments was observed in *B. subtilis* cultures after autoclave decontamination at 121 °C for 20 minutes. Foam appeared 10.5 hours into the cultivation, and the colour of the cell broth changed from beige colour of LB medium to light brown and brown at 10.5 and 20.5 hours post-inoculation, respectively. Changes in broth's colour during microbial cultivation usually arise from secretion of myriad chromogenic compounds and secondary metabolites by cells. During cultivation, the broth's pH dropped from 7 to 6.8 followed by a gradual rise to 8.3. Intriguingly, a pungent odour (similar to decaying organic matter) emanated from the cultures after 24 hours of cultivation, which subsequently, increased in pungency till the experiment was terminated 48 hours post-inoculation as many flies could be seen moving on or around the cotton plugs on each of the replicate cultures – thereby, posing a hygiene issue in the lab. Number of flies present positively correlated with odour pungency. The flies were also observed to be constantly probing for entrance into the cotton plugs at various locations, which was reminiscent of flies foraging for food; thus, suggesting that the odourous compounds might have behavior modifying effects. For comparison, flies were not attracted to cultures of other bacterial species – e.g., *E. coli* DH5α (ATCC 53868), *P. aeruginosa* PRD-10 (ATCC 15442) and *P. protegens* Pf-5 (ATCC BAA-477) – grown under identical conditions, even though odours (distinct from *B. subtilis*) was detected after onset of stationary phase in each culture. As per laboratory safety requirements, no food was consumed in the lab or disposed-off in the dustbins,

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and all bins were fully covered. Thus, presence of flies was unlikely due to smell of rotting food in bins. Nevertheless, since the orbital shaker was situated close to the laboratory door; periodic opening and closing of the door could have provided a pathway for fly entry. The cotton plugs remained dry and fitted tightly to the shake-flasks throughout the experiment, highlighting that broth droplets generated during orbital shaking could not have transferred *B. subtilis* to the cotton plugs. The rest of the article will outline possible interpretations and hypotheses concerning the observations made, and lines of inquiries for future work (Supporting information). Nevertheless, in the absence of more data, discussion of the possible mechanisms underlying the phenomenon will remain as educated guesses.
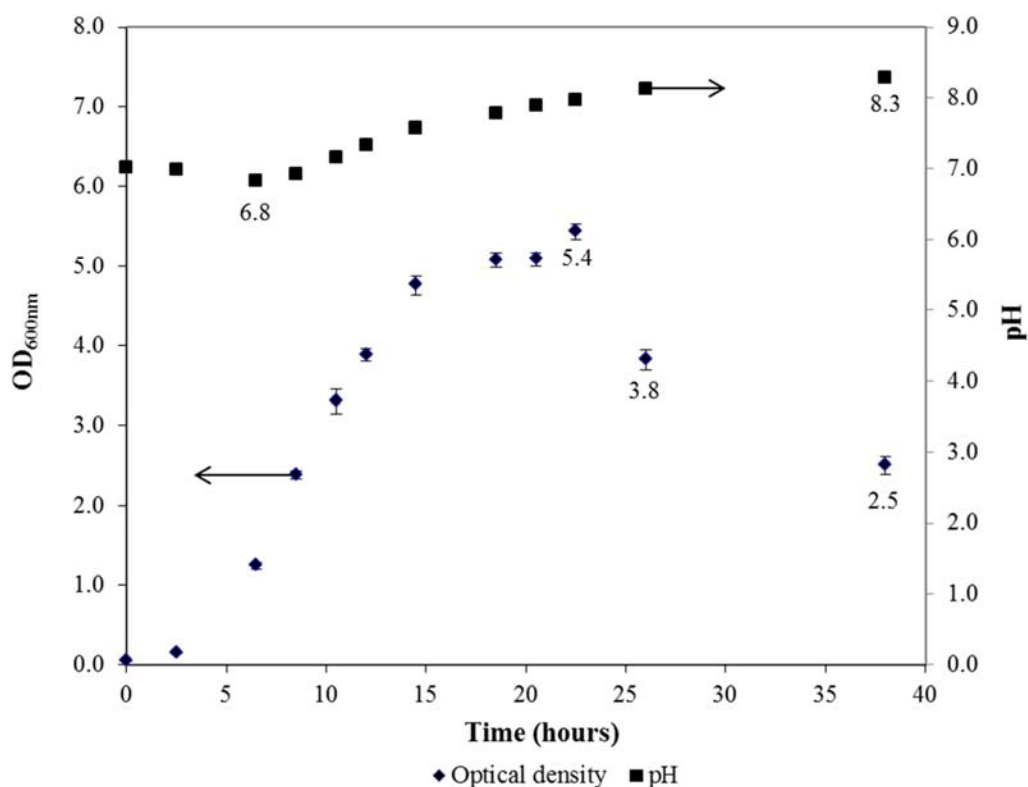

Fig. 1. Growth of *Bacillus subtilis* NRS-762 in LB Lennox medium at 25 °C and 250 rpm. Note the rapid decrease in optical density after attainment of maximum absorbance of 5.4 after 22.5 hours of cultivation.

First, without identity of the odour compounds and understanding of the molecular mechanisms by which they interact with the insect, a causal interkingdom signaling mechanism between flies and *B. subtilis* could not be defined at this juncture. The mode in which the flies behaved and moved on the cotton plug, however, might provide circumstantial evidence suggesting that the odourous compounds helped attracted flies to *B. subtilis*. The alternative

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explanation of presence of flies inducing the secretion of volatiles by the bacterium was possible, but given that odour pungency consistently correlated with fly presence, numbers and foraging behavior on the cotton plugs, fly-induced secretion of volatile compound(s) by *B. subtilis* would be unlikely. Nevertheless, confirmation of the directionality of casuation would require isolation of the attractant compound(s) and examination of the two hypotheses in controlled settings.

Rapid decline in OD_{600} during late stationary phase and absence of cell fragments after autoclave decontamination suggested that cell lysis might have occurred population-wide. Since cell lysis could be mediated by stress-induced activation of autolysin (during cannibalism) or, entry of prophages into the lytic cycle, the exact cause of the drastic decline in OD_{600} could not be pinpointed without further studies. More important, the onset of OD_{600} decline (~ 2.5 hours post-inoculation) coincided with emanation of pungent odour from the culture; thus, suggesting that release of cytoplasmic content might be the source of odourous compounds. Nevertheless, given that biosynthesis of odourous compounds would incur both a metabolic energy and precursor cost, why would the bacterium expend energy in synthesizing a signaling molecule prior to induction of cell lysis? What are other possible benefits to the bacterium of initiating such biosynthetic activity? More intriguingly, was *B. subtilis* NRS-762 synthesizing an odourous compound as an interkingdom communication agent in a desperate attempt to attract insects for helping disperse at least a subpopulation of the culture to more favourable ecological niches? The inverse relationship between odour pungency and OD_{600} may hold clues to the mechanism(s) underlying the phenomenon. Specifically, as OD_{600} declined precipitously, there was also a concomitant increase in odour pungency. While release of odour compounds through cell lysis might account for elevated pungency, the increased synthesis and secretion of volatile compounds by remaining cells as a SOS signal could not be ruled out. Finally, absence of cell debris in autoclaved cultures highlighted that sporulation might not have occurred or, proceeded to completion since spores are resistant to the high temperatures and pressures during autoclave decontamination.

The aforementioned "foraging" activities of flies on the cotton plug suggested that the volatile compounds exert possible behavior-modifying effects. If shown to be true in future studies, the observations would constitute evidence suggesting that bacteria could elicit desired behavior from an insect vector via direct modulation of cognitive behavior. Such a signalling mechanism would be significantly different from other known signalling modalities such as metabolite exchange, provision of essential nutrients to the host, and even modulating host immune response via metabolite secretion [29-33]. Taken together, the pungent odour emanating from the stationary phase culture could likely comprise a mixture of compounds with signaling, communication or behavior modifying functions.

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3.2   Phenotypic response of B. subtilis to nutritional stress

As a model microorganism for investigating bacterial multicellularity, *B. subtilis* engages in a variety of phenotypic and morphological transitions in response to differing severity and types of nutritional and environmental stressors. Specifically, *B. subtilis* explores a range of options involving cell differentiation (morphogenesis), induction of dormancy, cellular motility and secretion of antimicrobial or hydrolytic enzymes, all with the ultimate goal of ensuring that at least a subpopulation survives for propagating the clonal population [34]. Morphotypic transitions would include the known cell differentiation pathways of *B. subtilis*: i.e., cannibalism, sporulation, competence, and biofilm formation. On the other hand, induction of stringent response and persister formation are mechanisms that reduce cellular metabolic activity, which allows the bacterium to ride-out periods of low nutrient availability. Besides morphotypic transitions and entry into dormancy, *B. subtilis* also (i) activates flagellar motility for seeking alternative food sources via chemotactic mechanisms, (ii) secretes antibiotics and toxins for killing other microbial species competing for the same ecological niche, and (iii) uses hydrolytic enzymes for scavenging residual extracellular proteins and polysaccharides – an option of particular relevance to low nutrient (oligotrophic) environment [34]. Secretion of antimicrobial compounds is more relevant in a multi-species community context, but the same mechanism is also used by *B. subtilis* for lysing a fraction of the population in providing nutrients to the remaining cells.

Faced with progressive depletion of nutrients in stationary phase, *B. subtilis* initiates several differentiation programmes for creating subpopulations of cells with distinct metabolic activities and phenotypes. By generating subpopulations each suited to different trajectories in which environmental and nutritional conditions of the habitat could evolve, the probability that at least a fraction of the original population could cope with fluctuations in a microcosm's habitability significantly improves. In the case of prolonged decline in environmental conditions, transformation of vegetative cells into spores robust to a variety of harsh conditions brings the population into dormancy, but capable of regenerating a clonal population should more favourable conditions return.

One strategy employed by *B. subtilis* is to increase the available nutrient pool through the selective lysis – via endotoxins – of a fraction of the population in a process known as cannibalism [34-36]. Specifically, cannibalism is active at temperatures ranging from 32.5 to 40 ℃ but not at 20 ℃ [35]. Nevertheless, cannibalism is a possible differentiation pathway available to *B. subtilis* at 25 ℃ since the toxin responsible for cannibalistic behavior is synthesized at 20 ℃ [35]. Thus, the observed precipitous decline in optical density could be partially explained by induction of cannibalism with the resulting release of cytoplasmic content accounting, in part, for the increase in odour pungency.

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Another possible co-occurring differentiation pathway is competence, where the cell wall and membrane of *B. subtilis* vegetative cells becomes more permeable towards a variety of molecules – particularly extracellular DNA [34,37,38]. Both active and passive uptake of DNA from the extracellular environment increases the probability of augmenting cellular metabolic capabilities; for example, in catabolizing remaining nutrients in the habitat that the endogenous metabolic repertoire is naïve to. DNA obtained from the environment could also be used for repairing endogenous DNA sequences encoding functions for coping with environmental stressors [34]. But, more directly, extracellular DNA is a ready source of carbon and nitrogen that a nutritionally stressed cell population can subsist on. Nevertheless, if conditions continue to deteriorate – such as imminent depletion of nutrients - entry into the sporulation pathway would have to be initiated [35,36]. Thus, both cannibalism and competence are pathways leading to sporulation, regulated by a cellular decision checkpoint; for example, after spending several hours in the competent state, cells switch back to vegetative growth before final entry into sporulation [34].

Spore formation is effective for ensuring population survival under the most severe environmental stressors given their low basal metabolism and resilience against harsh environmental conditions (temperature, pressure, desiccation, and radiation), but the high metabolic cost (activation of >500 genes over 10 hrs) and irreversibility of the differentiation programme meant that progress down the sporulation pathway is the last option [34]. Thus, a commitment stage exists as a checkpoint for making the lineage differentiation irreversible in case more favourable conditions return [34,36,39].

Depending on the timescales involved, the range of survival options can be categorized into those designed for coping with short-term fluctuations in environmental conditions or ones catering to long-term irreversible decline in habitability. Under this framework, approaches for inducing dormancy (such as persistence, stringent response etc.) are short-term responses suitable for returning the population to vegetative growth once favourable conditions return. In contrast, spore formation are evolutionarily selected for seeding a residual population to cope with long-term (e.g., 10^1 to 10^3 years) deterioration of habitability. Irrespective of the timescales, the aforementioned survival strategies are *in situ* mechanisms, since the differentiated dormant cells or spores are likely to remain in the vicinity of the original habitat without wind or vector mediated long-range dispersal.

In addition to classifying survival mechanisms based on the dominant timescale at which effects manifest, stress responses could potentially be categorized via their dependence on vector-based dispersal. Besides the "static" (or *in situ*) approaches outlined above, which essentially

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involves the species waiting out a crisis in hope of better environmental conditions to return at the same site in future, another possibility would be to actively find ways of dispersing a subpopulation of cells away from the current habitat (e.g., the shake-flask), hopefully to a new ecological niche conducive for growth. Pursuing the more proactive approach would necessitate finding a transport vector – for example, flies or mosquitoes – by establishing a line of communication between the bacterium and vector, probably via signaling molecule(s). More important, from the ecology perspective, the vector should find the bacterium palatable – and, more preferably, confer some benefit to it. An essential feature of this schema is the long foraging range of the vector relative to that of the bacterium, which provides a higher probability of long-range dispersal to sites with more favourable growth conditions.

Given that *B. subtilis* is a commensal in many fly species, the general idea that flies might have served as a transport vector for the bacterium in exchange for metabolic benefits is plausible [40]. Such benefits may include preventing the colonization of the fly's gut by invasive bacterial species, or augmenting the fly's metabolic repertoire through the provision of essential nutrients and vitamins [41]. Recent research indicates that the gut microbiome of insects may comprise a "core" and "transient or context-dependent" segment, where the core residents provide essential functions unavailable to host cells, while the transient population augments the metabolic capabilities of the host depending on the prevailing nutritional or environmental conditions [29]. Such a microbiome community structure enables the host to rapidly respond to changes in nutrient availability.

3.2.   *Conceptual model*

Holistic analysis of the preliminary observations suggested that *B. subtilis* NRS-762's entry into stationary phase might have initiated the biosynthesis of odourous compound(s), which attract flies as transport vectors for dispersing the bacterium to new ecological niches (Fig. 2). Naturally, the preferred choice would be as far away from the original habitat as possible since localities in close proximity tend to share similar environmental conditions/stressors. Concomitantly, cell lysis was initiated – either mediated by cannibalism or lytic phages or both, which resulted in an observed drastic decline in OD_{600} that culminated in a probable collapse of the entire population, since no cellular debris was observed in the shake-flasks after autoclave decontamination. The observation also strongly suggested that *B. subtilis* NRS-762 did not complete sporulation during stationary phase. More intriguingly, the odour increased in pungency, while OD_{600} declined precipitously between 24 and 48 hours post-inoculation, which suggested secretion of volatile compounds could be cell density-dependent. In particular, upon sensing declining cell numbers, a cell density-based signalling mechanism induce the secretion of more volatile compounds by remaining cells in a positive feedback loop, probably as a desperate survival mechanism.
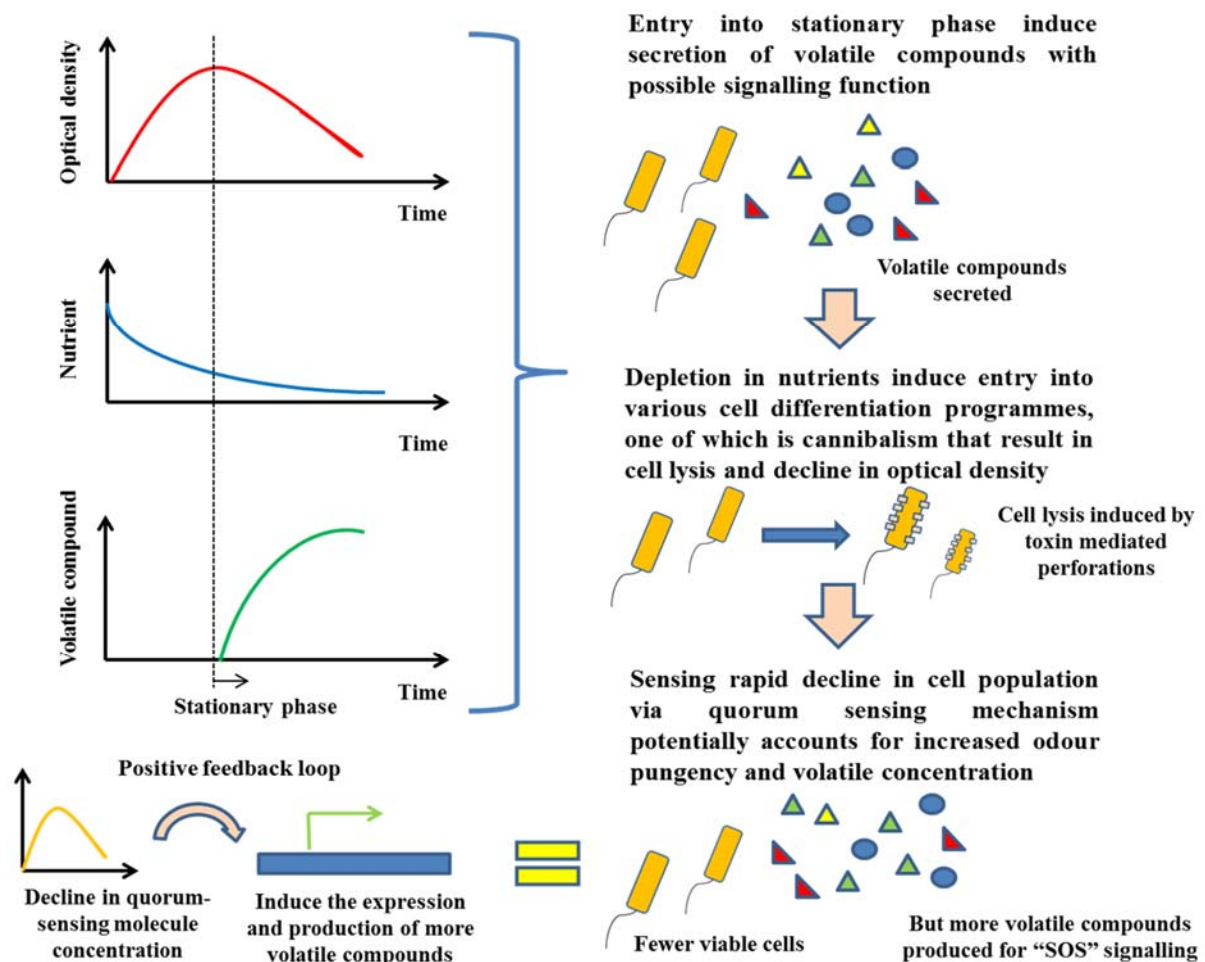
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Fig. 2. Conceptual model illustrates how nutrient depletion during stationary phase could have induced interkingdom signalling between flies and *B. subtilis* NRS-762 for long-range dispersal of the bacterium to alternative ecological niches. Specifically, a hypothesized mechanism linking cell density decline and increase in volatile compound production and secretion to vector-based dispersal to more favourable ecological niches was presented.

Dispersal of *B. subtilis* to a new habitat provides a means for a probabilistic search of more favourable ecological niches in propagating a new clonal population, given the depletion of nutrients in the shake-flasks. In return for the transport service, *B. subtilis* might confer metabolic benefit to the fly through colonization of the mucosal surfaces in the gut or help protect the resident microbiota against invasive or pathogenic microbial species. Intriguingly, the observations also highlighted possible existence of an alternative coping strategy of *B. subtilis* during times of nutritional stress, where secretion of odourous bioattractants for signalling long-range dispersal occurs in parallel with more static (*in situ*) approaches such as induction of cannibalism or sporulation (Fig. 3).
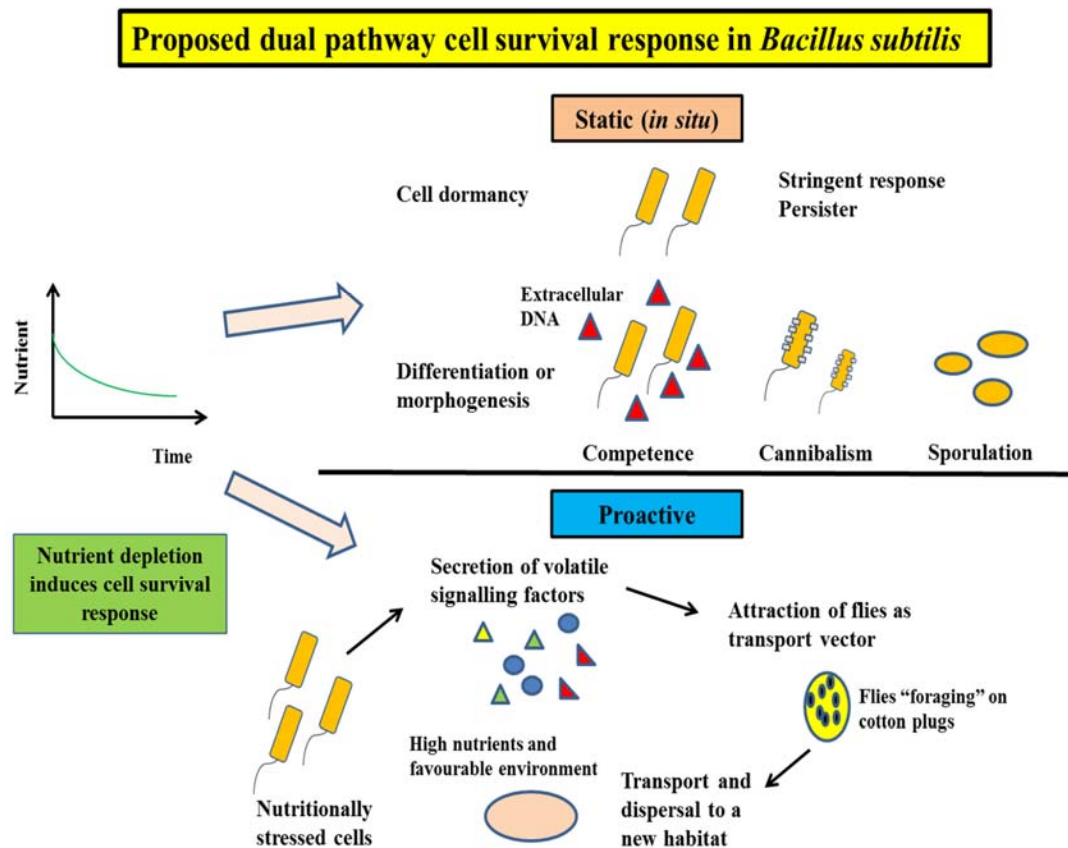
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Fig. 3. Depiction of a possible two-prong or dual pathway survival strategy in *Bacillus subtilis*. Specifically, upon nutrient stress, both "static (*in situ*)" and "proactive" survival responses are induced in parallel, where, in the former, various cell dormancy and differentiation pathways are activated to generate multiple subpopulations of cells for responding to short- and long-term fluctuations in environmental and nutritional conditions. On the other hand, "proactive" responses postulated in this study involve the secretion of volatile signaling factors for attracting flies (or other insect vectors) in transporting the bacterium to ecological niches with better habitability. The approach would be useful for coping with potential irreversible decline in habitability of the existing microcosm. Combined, the twin approaches afford *B. subtilis* expanded capabilities for surviving myriad environmental stressors occurring across a range of temporal and spatial scales, which, from an evolutionary perspective, may help account for the versatility, adaptability and ubiquitous nature of this benign bacterial species, whose natural habitat is the soil environment.

In contrast to the "static" mechanisms described above, volatile compounds mediated long-range dispersal of *B. subtilis* to more favourable locales can be christened as a "proactive" response to environmental stressors. In this framework, cannibalism helps *B. subtilis* respond to temporary fluctuations in nutritional stress without committing to the energy-intensive process of sporulation. But given the significant time required for spore germination and outgrowth into vegetative cells,

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unnecessary sporulation would reduce *B. subtilis* fitness in competing with other species in the same habitat should an uptick in environmental conditions occur shortly thereafter. Generally, spore formation is more suited for responding to potential long-term decline in environmental conditions on a seasonal or longer timescale. On the other hand, "proactive" responses are ideally suited to scenarios such as irreversible decline in habitability. In general, closely-separated locales tend to share similar nutritional and environmental conditions. Thus, by inference, localities further away from the original habitat would have a higher probability of having more favourable conditions. Hence, given the evidence presented in this research note, it is possible that *B. subtilis* NRS-762 hedges its bet on survival by simultaneously pursuing the twin strategies of initiating cannibalism (a static response), and long-range species dispersal via interkingdom signaling and vector mediated transport (a proactive response, Fig. 3).

Lack of appreciation of the serendipitous observations' significance meant that direct evidence and data are not available for supporting the claims made. Analysis of the direct and circumstantial evidence, however, helped formulate a conceptual model postulating physiological underpinnings of the observed phenomenon and its ecological implications. The model posited that there exists, in parallel to the known processes of cannibalism and sporulation, an alternative pathway where *B. subtilis* seek to attract transport vectors (such as flies) for long-range dispersal of a subpopulation to more habitable ecological niches. Such dispersal would improve the population's chances of survival in the event that the original habitat becomes uninhabitable. From a different perspective, adaptation approaches such as induction of cannibalism and sporulation can be viewed as static (*in situ*) methods for riding out temporary fluctuations in environmental stressors – where likelihood exists for more favourable conditions to return in the future. Nevertheless, the possibility that environmental conditions may be on a permanent downward trajectory, or fluctuations in conditions becoming more intense and frequent exists, for example, as a result of climate change. Thus, selective pressure likely exists for the evolution of a two-prong strategy where bacteria attempt to at least disperse a subpopulation to regions further afield, and execute cellular differentiation programmes for generating subpopulations more suited for overcoming short- to medium-term fluctuations in environmental conditions via dormancy mechanisms. Finally, caution should be exercised in interpreting the aforementioned preliminary observations since they were made with a pure culture system. Specifically, given that substantial metabolic energy must be expended in synthesizing and secreting the odour compound(s), the utility of attracting flies may not be as apparent in the mixed culture case since the flies may ingest and disperse other bacterial species (i.e., free-riders) instead of the one secreting the bioattractant.

The preliminary observations presented could be extended in a number of directions after experimental verification of the phenomenon (details are in Supporting Information). Examining whether odour evolution and associated attraction of flies occur across growth media and other *B.*

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subtilis strains or *Bacillus* species would constitute the first steps in understanding the specificity of the phenomenon. Given the centrality of odour compounds in mediating potential interkingdom signaling between bacteria and flies, profiling the compendium of volatile compounds via mass spectrometry techniques would provide the molecular identities important for investigating structural aspects important for molecular recognition – and similarity of the signaling mechanisms to other known pathways. The first steps in this endeavor could be the direct comparison of the profiled compounds with volatile compounds known to partake in inter-species and interkingdom signaling. Subsequently, the species specificity of individual compounds could be determined, which would help shed light on the prevalence (and extent of conservation) of particular signaling modality across different branches of the tree of life – with the possibility of identifying new signaling pathways or hitherto unknown functions of known signalling molecules. While establishing the molecular players involved in interkingdom signaling provide the overall framework for understanding the pathways mediating information transfer, elucidating the ecological significance of the delineated processes would afford the connection of molecular details to ecosystem interactions. Efforts in this direction may examine the roles of ingested bacteria in preventing colonization of the insect's gut by pathogenic species and maintaining the gut microbiome in the "healthy" state, or providing metabolic capabilities absent in the host. Data obtained would help define the ecological relationships between two species and, more important, the underlying functions and chemical basis. Finally, studies could be conducted to identify the feedback processes (and associated signaling and metabolic pathways) mediating odour compound secretion and cell population decline. Knowledge gleaned from such studies would aid our understanding of cell differentiation pathways in the metabolically versatile and resilient bacterium, *B. subtilis*, and the complex processes underlying cellular decision-making at the molecular level.

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